THE REMOVAL OF DIVALENT CATIONS FROM SOLUTION BY BEEF HEART ANTIGENS[1]

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The exact mechanism of the precipitation-flocculation tests has been studied for nearly thirty-five years by investigators throughout the world. Of many explanations offered, the most accepted is that a substance called "reagin" in blood sera reacts with the lyophilic colloid, antigen, causing it to become a lyophobic colloid and thereby produce a floc. Much work has been done in trying to isolate this "reagin" of syphilis with little or no result.

In the last five years the greatest advancement made in serodiagnosis of syphilis has been, not in improved techniques or methods, but in that the clinician has come to realize that diseases other than syphilis can rather frequently yield positive test results. Porro (1) has shown that certain animals apparently possess the so-called "reagin" in their blood normally. Greene and associates (2) later confirmed Porro's work and showed that there were a great many other animals that would react positively towards the standard syphilis tests used today. Later Breazeale, Reusser and Breazeale (3), and Reusser, Breazeale and Breazeale (4) showed that saps from the tops of many plants react positively to commonly used tests, while saps from the roots fail to flocculate these standard antigens. These investigators were able to dilute saps from the top of the plants as high as 1 to 256 before they failed to flocculate the antigens. Greene and Breazeale (5 and 6) have studied the reaction in horse and cow sera. They have shown that titers in these animals ran as high as 1:64 and 1:128. Breazeale (7) has been able to shift reactivity of sera from positive to negative by the use of ultraviolet irradiation. Breazeale, Reusser and Breazeale (8) succeeded in shifting sero-negative sera to sero-positive, and vice versa, by the treatment with pure zeolites. Breazeale, Pierce and Breazeale (9), have been able to adsorb "reagin" out of syphilitic serum by the use of Kahn and Hinton antigen, thereby producing a sero-negative serum. By treating this experimentally produced sero-negative sera with calcium zeolite they were able to again obtain positive flocculation reactions to the various tests. Later they (10) were able to apply this same principle to a test for syphilis by employing a suspension of pure sodium zeolite as an indicator.

Since it has been possible to reverse serologic reactions of the sera by use of a zeolite, it seemed probable that the presence of some cation was responsible for production of a floc. Further, it was possible to obtain flocculation of pure sodium zeolite by syphilitic sera and not by normal sera, and also by normal sera

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treated with calcium zeolite. This suggested that antigen, as used in serology today, functions as a zeolite. With this in mind the following experiments were undertaken.

EXPERIMENTAL

Blood samples as received at the laboratory were separated from the clot by centrifugation at 4,000 r.p.m. The clear supernatant sera was then drawn off, pooled without regard to their reactivity, and divided into four portions. One portion was used to determine the calcium content in the sera. To the second and third portions Kahn and Hinton antigen were added in the proportion of 1:3. The tubes were shaken for ten minutes and centrifuged for an additional ten minute period. The clear supernatant sera were withdrawn and the calcium contents determined. Sodium zeolite was added to the fourth portion of the sera so that there was 10 per cent by volume of pure zeolite added to the sera.

TABLE I

The removal of calcium from human sera by Kahn antigen, Hinton antigen and sodium zeolite (50 sera)

	CONTROL	CALCIUM REMAINING IN SERA AFTER ADSORPTION WITH:		
		Kahn antigen	Hinton antigen	Sodium zeolite
	mgm.	*mgm.*	*mgm.*	*mgm.*
Average.....	10.5	4.0*	4.0*	4.5*
Maximum.....	12.0	5.0	5.0	5.5
Minimum.....	8.8	3.5	3.5	3.5

* Calculated to original volume of sera.

The tubes were then shaken for ten minutes and centrifuged as above. The clear sera were then drawn off and calcium determinations run on them. The results are given in Table I. A total of fifty sera were run in this manner. The average values, maximum and minimum values obtained, are given in the table expressed as milligrams of calcium per 100 ml. of sera.

It will be noted that an average of 6.5 mgm. of calcium was removed from the sera by both the Kahn and Hinton antigens and 6 mgm. by the zeolite.

Three samples of sodium zeolite were treated with (a) $AlCl_3$, (b) $FeCl_3$ and (c) heat, thereby "killing" them, that is, destroying their power of base exchange, and the above experiments repeated. The "killed" zeolites did not adsorb any calcium and the treated sera gave identical calcium contents with the control. The calcium removals obtained with Kahn and Hinton antigen treated sera were the same as for the first experiment given in Table I. This experiment indicates strongly that the active agent in the sera was a divalent cation.

An aqueous solution of calcium sulfate and magnesium sulfate was made up so as to contain 309 parts per million each of Ca and Mg. Fifty samples of ten ml. each were measured into 15 ml. centrifuge tubes, and two ml. standard Kahn antigen was added to each tube. The samples were shaken for a period of three

minutes, centrifuged at a speed of 4,000 r.p.m. for ten minutes, and eight ml. of clear supernatant fluid, representing 1.85 mgm. each of Ca and Mg, drawn off. The calcium and magnesium were determined by the soap titration method as outlined by Schriener and Failyer (11) (this method was checked by Breazeale and Greene (12) and found accurate and precise). The experiments were repeated using Hinton antigen in place of Kahn antigen with identical results. The results are given in Table II.

A third set of experiments was set up as follows. Twenty ml. of a standard solution of calcium sulfate and magnesium sulfate containing 309 p.p.m. of each cation were measured into large test tubes and 2 ml. of standard Kahn antigen added to each tube. The tubes were shaken for three minutes and then the

TABLE II

The removal of calcium and magnesium from aqueous solution by Kahn and Hinton antigens (50 samples)

	Ca added	Ca recovered	Ca removed	Per cent Ca removed by antigen	Mg added	Mg recovered	Mg removed	Per cent Mg removed by antigen
	mgm.	*mgm.*	*mgm.*		*mgm.*	*mgm.*	*mgm.*	
Average	1.85	1.00	0.85	47	1.85	0.65	1.20	65
Maximum		1.10	0.9	49		0.70	1.25	68
Minimum		0.95	0.75	41		0.60	1.15	62

TABLE III

The removal and recovery of calcium and magnesium from aqueous solutions (50 samples)

	Distilled H_2O from filter paper		Mg in original solution		Mg in adsorbed filtrate		Mg recovered from floc	
	Ca	Mg	Ca	Mg	Ca	Mg	Ca	Mg
Average	0	0	3.09	3.09	2.05	1.8	0.8	1.05
Maximum	0	0			2.10	1.9	1.0	1.25
Minimum	0	0			2.00	1.7	0.7	1.00

solution filtered through washed ashless filter papers. The filtrate from Kahn absorbed solution was collected and 11 ml. used to determine the Ca and Mg. After the filter papers had been allowed to drain for ten minutes they were washed on the funnel first with distilled water and then with 10 ml. of 1 per cent sodium chloride solution and this last filtrate analyzed for Ca and Mg by soap titration.

If the antigen was acting in the role of a mono-valent cation zeolite one would expect that divalent cations would be taken out of solution by the floc. And, if one washed the floc on the filter paper with sodium chloride solution, divalent cations would be replaced by the sodium. Thus their presence in the filtrate could then be determined quantitatively. These results are given in Table III. In order to show that no calcium or magnesium was obtained from the filter

papers themselves, all papers were washed with 10 ml. of distilled water and the calcium and magnesium content determined in the filtrate by soap titration.

A fourth set of experiments was set up as follows. Pooled negative sera was divided into four samples. Sample number one was examined by the standard Kline and Kahn techniques, and calcium determined by precipitation as the oxalate and titrated with potassium permanganate. The second sample was treated with calcium zeolite as previously described and the clear supernatant sera examined by the methods of Kline and Kahn, and the calcium content determined as above. The third sample was treated with sodium zeolite and likewise examined. The fourth sample was absorbed with standard Kahn antigen in the ratio of 1:3, centrifuged and the clear supernatant fluid examined by Kline and Kahn methods, and calcium content determined and expressed in terms of original serum. The results are given in Table IV.

TABLE IV

The effect of treating pooled sera with CaZ, NaZ and Kahn antigen on the sero-activity and calcium content (20 samples)

TREATMENT	REACTION TO:		MILLIGRAMS OF Ca PER 100 ML. OF SERA
	Kahn	Kline	
			mgm.
No treatment	Negative	Negative	10.0
CaZ treated	4 plus	4 plus	80.0
NaZ treated	Negative	Negative	4.5
Kahn antigen adsorbed	Negative	Negative	3.5

The last experiments were repeated using NaZ and CaZ which had been "killed" by heat, aluminum chloride and ferric chloride. None of these shifted either calcium content nor reaction to the two tests for syphilis.

DISCUSSION

The mechanism of the various flocculation tests for syphilis has long been investigated and many answers and reasons have been given for its reaction. The most widely accepted explanation is that there is a substance called "reagin" in the sera that reacts with the lyophile colloid, antigen, thereby forming a lyophobic colloid which flocculates. However, "reagin" is not specific for syphilis and is found in the sera of patients suffering from numerous other diseases, notably those giving rise to fevers.

Examination of results obtained in these experiments as outlined here reveal interesting facts. It is obvious that the so-called "reagin" is a divalent cation, either that of calcium or magnesium, or both. Since calcium exists in blood to the extent of from 9 to 12 mgm. per 100 ml. of blood, and magnesium to the extent of only between 1.5 to 3.0 mgm. per 100 ml. of blood, it would seem that calcium would be the principle ion affecting the tests rather than magnesium.

In Table I calcium determinations on blood sera were run before the sera

was treated in any way, and also after adsorbing with Kahn and Hinton antigens and sodium zeolite. It will be noted in this table that standard antigens removed an average of 6.5 mgm. of calcium (62 per cent), while sodium zeolite removed 6.0 mgm. of calcium per 100 ml. of sera (57 per cent). These experiments were repeated with "killed" zeolites with no removal of calcium whatsoever. This factor would indicate that removal of calcium from blood sera was a function of base exchange.

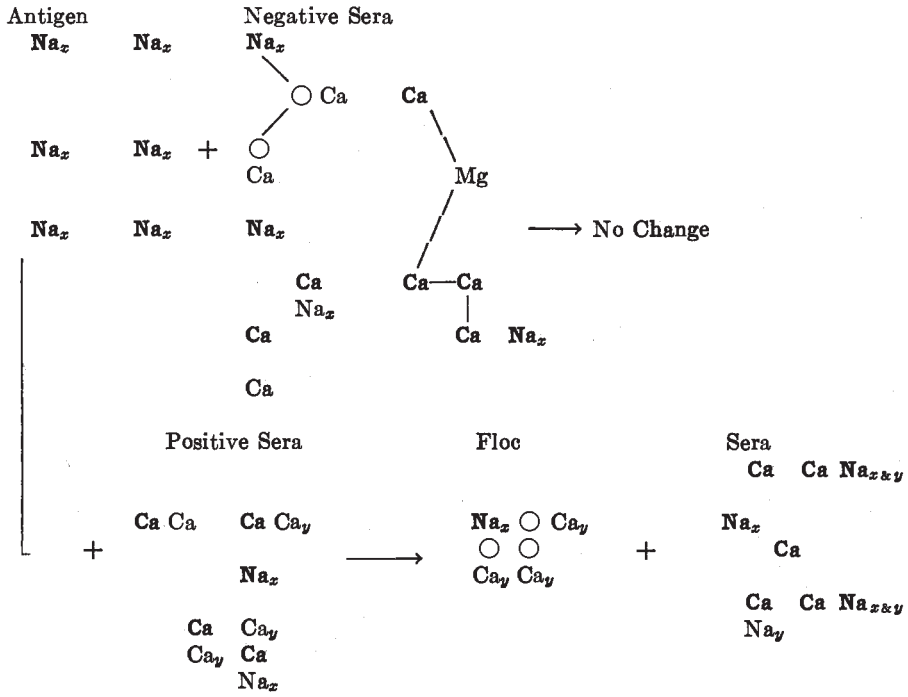
From common knowledge of zeolites and zeolitic replacement we know that one base may be replaced by another providing the first is in higher concentration than the second, or has a higher replacement value than the second. That is, we know that calcium is one of the bases with high replacement value, but that it may be replaced from the zeolitic complex by sodium if the monovalent base is in predominance. In Table III with Kahn antigen we adsorbed calcium and magnesium from a known solution containing a mixture of equal parts of calcium and magnesium, and removed the floc by filtration through an ashless filter paper. The calcium and magnesium had been removed from the solution by the antigen as was evidenced by the fact that we had a decrease in the total amount of calcium and magnesium in the filtrate. After washing with distilled water the precipitate was then washed on the filter paper with a 1 per cent solution of sodium chloride. If calcium was bound to the antigen in a stable chemical compound one would not expect to be able to recover any calcium or magnesium in the filtrate from these washings. If, however, the cations were chemically combined into a fraction possessing base exchange properties it would follow that they could be replaced by sodium ions. This was the case as shown in Table III. This fact would strongly indicate that beef heart antigen acts purely as a zeolite and therefore possesses base exchange properties. McGeorge (13) has definitely shown that various organic matters may exchange their bases for others in the same manner as true zeolites, therefore it is not surprising that usual beef heart antigens function as such.

Finally, if we could show that a serum became positive when treated with calcium zeolite and had an increase in calcium content, and that when we treated a serum with Kahn antigen or sodium zeolite its calcium content was reduced, it would further tend to prove that antigen was purely an organic substance acting in base exchange capacity. This was clearly brought out in the experimental results given in Table IV. There was a great increase in the calcium content in sera treated with calcium zeolite (80 mgm./100) and such sera reacted positively to the Kline and Kahn tests. Those sera treated with Kahn antigen and sodium zeolite showed a sharp decrease in calcium content (3.4 and 4.5 mgm./100) and also, they gave negative Kahn and Kline tests.

These experiments were repeated using zeolites "killed" by various methods, by saturating with Al^{+++}, Fe^{+++} and also by heating to a dull redness for ten to thirty minutes. In every case sera treated with these "killed" zeolites showed no change in serologic reactions or their calcium or magnesium contents, while parallel samples treated with standard Kahn and Hinton antigens showed a definite lowering of calcium and magnesium contents. This factor shows that

the removal of calcium and magnesium was due to zeolitic action of the active zeolites and also base exchange power of the usual antigens. Original reactivity of sera seemed to have no effect on the amount of calcium removed.

Since the original reaction of sera seemed to have little or no effect on removal of calcium by antigen or zeolites, it would seem that production of a floc depended upon actual ionic concentration of the divalent cations in solution. Therefore, the following schematic representation may be used to represent the reaction.



Antigen in this diagram is represented as a zeolite that is saturated with sodium. Since we dilute our antigen with physiological saline, the lipoid must carry sodium in its outer shell. We add to this antigen a serum high in calcium of which about two to three mg. per 100 ml. is in the ionic state. This ionized calcium immediately replaces the sodium and thereby is removed from the field of action. On the other hand in syphilitic sera there must be more calcium in the actual ionic state or the ionization constant must be greatly altered and therefore it reacts more readily with the zeolite. Thereby, a calcium zeolite is formed more rapidly, producing larger flocs and the typical floc of a positive test appears.

It will be noted that the reaction, as diagrammed, depends upon the presence of ionic (or rapidly available ionically), calcium or magnesium. At the same time it is noted from results obtained here that calcium was removed as readily from positive sera as from negative sera. Therefore it appears that the produc-

tion of a floc depends upon the degree of ionization of calcium and not merely upon its molecular availability. That is to say that there must be either an increase in ionization of divalent cations or some profound vectorial shift with respect to the ionization constant of calcium compounds in sero-positive sera which causes the antigen to floc. Surely beef heart antigens today are acting purely in the capacity of a zeolite and shift from a sodium salt to either a calcium and/or magnesium salt thereby producing a floc.

SUMMARY

1. Calcium and magnesium were removed from aqueous solutions by means of Kahn and Hinton antigens.

2. Calcium and magnesium were removed from solution by Kahn antigen and the floc removed by filtration. The floc was then washed with saline, and the calcium and magnesium recovered in the filtrate.

3. A new theory of the mechanism of syphilis serology is advanced.

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